Alkali cation permeability and caesium blockade of cromakalim-activated current in guinea-pig ventricular myocytes

¹J.C.R. Randle, ²S.H.R. Olié & J.-F. Renaud

FONDAX, Groupe de Recherche Servier, 7, rue Ampère, 92800 Puteaux, France

1 The sensitivity of cromakalim-activated current (I_{crom}) to manipulations of extracellular cationic composition was examined in whole-cell voltage clamp recordings from freshly-dispersed, adult guinea-pig ventricular myocytes. In bathing media with different concentrations of K^+ (1, 2.5, 5.4 and 12 mM) the I_{crom} reversal potential (E_{rev}) varied in strict correspondence with the K^+ equilibrium potential and inward I_{crom} amplitude was proportional to the external K^+ concentration.

2 Replacement of 12 mM K^+ with 12 mM Rb^+ induced a slight positive shift of E_{rev} indicating that $P_{\text{Rb}^+}/P_{\text{K}^+} = 1.06$. K^+ replacement with 12 mM Cs^+ reduced or abolished inward I_{crom} and produced a negative shift of E_{rev} by at least 50 mV; an upper limit of $P_{\text{Cs}^+}/P_{\text{K}^+}$ was fixed at 0.18.

3 Addition of Rb^+ (1–30 mM) to 2.5 mM K^+ -containing medium produced a concentration-dependent increase in inward I_{crom} and positive shift of E_{rev} suggesting that K^+ and Rb^+ have similar permeabilities and conductivities and do not interfere with each other in the channel.

4 Cs^+ (0.01–30 mM), added to medium containing 12 mM Rb^+ , induced a potent, voltage-dependent inhibition of inwardly rectifying current (I_{K1} ; $\text{IC}_{50} = 0.2$ –3 mM). Voltage-dependent inhibition of inward I_{crom} was observed only at considerably higher Cs^+ concentrations ($\text{IC}_{50} = 4$ –30 mM). Extracellular Rb^+ and Cs^+ did not substantially alter the amplitude of outward I_{crom} .

5 The results support the contention that the ATP-sensitive K^+ channel is the primary target of cromakalim action in ventricular myocytes.

Keywords: ATP-sensitive K^+ channel; whole-cell patch clamp; inward rectifying K^+ channel I_{K1} , Rb^+ ; voltage-dependent block

Introduction

Cromakalim is a benzopyran derivative that activates ATP-sensitive K^+ [K(ATP)] channels in cardiac myocytes (Escande *et al.*, 1988; Sanguinetti *et al.*, 1988), arterial smooth muscle cells (Standen *et al.*, 1989) and an insulin-secreting cell line (Dunne *et al.*, 1989). Since the relaxation of smooth muscle and decrease of blood pressure that it induces are inhibited by sulphonylureas such as glibenclamide (Standen *et al.*, 1989; Quast & Cook, 1989a; Winquist *et al.*, 1989; Caverio *et al.*, 1989; Eltze, 1989) which block K(ATP) channels (Schmid-Antomarchi *et al.*, 1987; Sturgess *et al.*, 1988), K(ATP) channel activation may be the primary mechanism of action of cromakalim. A complementary approach to examine this mechanistic link in electrophysiological experiments is to compare membrane permeability changes induced by cromakalim in whole-cell recordings with the permeability of the K(ATP) channel. Single channel studies of the ionic selectivity of K(ATP) channels in heart, skeletal muscle and β -pancreatic cells indicate that they are highly permeable to K^+ and Rb^+ but not to Na^+ (Kakei *et al.*, 1985; Spruce *et al.*, 1987; Ashcroft *et al.*, 1989) and are blocked by Cs^+ (Quayle *et al.*, 1988). In this paper we describe the effects of changes in extracellular alkali cation composition on cromakalim-activated current (I_{crom}) in whole-cell recordings of adult guinea-pig isolated ventricular myocytes.

Methods

Preparation of adult guinea-pig ventricular myocytes

Ventricular myocytes were isolated by a technique adapted from Mitra & Morad (1985). The two principal modifications

to the technique were, (a) the use of a peristaltic pump to regulate flow perfusion medium at 6–8 ml min⁻¹ through the heart and, (b) the gradual reintroduction of CaCl_2 to the isolated cell suspension at 5–10 min intervals to intermediate concentrations of 100 μM , 200 μM , 500 μM and 1 mM. Isolated myocytes were stored at 37°C and used within 6 h.

Whole-cell patch clamp experiments

Electrophysiological measurements were made by the whole-cell patch clamp technique (Hamill *et al.*, 1981). The cells were placed in a plexiglas recording chamber maintained at 33–35°C and perfused with medium of composition (in mM): NaCl 143, KCl 2.5, MgCl_2 1, CaCl_2 1.8, NaH_2PO_4 0.33, N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) 10 and glucose 10, at pH 7.35. Recording pipettes were made from 1.2 mm borosilicate glass tubing and filled with a solution containing (in mM): KCl 140, NaCl 1, MgCl_2 2, CaCl_2 3.3, HEPES 10, ethyleneglycol-bis (β -aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) 10, diNa⁺ adenosine 5'-triphosphate (ATP) 3 and Na⁺ adenosine 3':5'-cyclic monophosphate (cyclic AMP) 0.1, adjusted to pH 7.15 with ~3 mM KOH and had tip resistances of 4–8 M Ω . A 4% agar/NaCl bridge was used to connect the Ag/AgCl ground wire to the bath solution and variation of junction potential associated with solution changes was never greater than 1 mV. Membrane currents were recorded by use of a Biologic RK300 amplifier with careful series resistance compensation.

Voltage protocol generation and data acquisition and analysis was achieved by use of an A–D converter (Labmaster DMA, Scientific Solutions) and interface (TL-1, Axon Instruments) and a microcomputer (IBM PC-AT) equipped with an appropriate software package (pCLAMP, Axon Instruments). Data were digitized at 1–2 kHz. Averaged data from several cells were expressed as the mean $\pm$ standard error of the mean (s.e.mean). Statistical treatment of data was performed by analysis of variance followed by the Newman-Keuls test.

¹ Author for correspondence.

² Present address: Centre for Research in Neuroscience, Montreal General Hospital Research Institute, 1650 Cedar Avenue, Montreal, Quebec, H3G 4A1, Canada.

Cromakalim [(–)-BRL 34915; (–)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo [b] pyran-3-ol], synthesized in the chemistry department of the Groupe de Recherche Servier, was prepared as a stock solution (10, 50 or 100 mM) in dimethylsulphoxide (DMSO) stored at +4°C, diluted to 100 μ M in the appropriate medium just before use and applied by superfusion. In control experiments, the DMSO vehicle solution (0.1–1%) had no effect on ventricular myocyte membrane currents. Changes in the ionic composition of the perfusion medium were made as follows. Different K^+ concentrations (1–12 mM) were obtained by equimolar replacement of NaCl with KCl. When RbCl or CsCl (12 mM) was added, KCl was omitted and NaCl was reduced from 143 mM to 133 mM. In the remaining experiments, RbCl (1–30 mM) or CsCl (0.001–30 mM) were added to the bathing medium and an equal amount of NaCl was removed from normal medium containing 2.5 mM KCl or 12 mM RbCl.

Results

Cromakalim-activated current (I_{crom}): dependence upon extracellular potassium concentration ($[K^+]_o$)

Whole-cell membrane currents were measured during 100–200 ms voltage steps over the range –200 mV to +80 mV. Under control conditions (before application of cromakalim), guinea-pig ventricular myocytes display a prominent inward K^+ current (I_{K1}) that varies in amplitude and voltage-dependence with $[K^+]_o$. The results illustrated in (Figures 1 and 2a) are very similar to those reported previously in cardiac (Sakmann & Trube, 1984; DiFrancesco *et al.*, 1984; Tournier *et al.*, 1987) and other preparations (Hagiwara & Takahashi, 1974; Leech and Stanfield, 1981). A relatively small outward K^+ current is also evident at positive potentials (see below) and inward Na^+ and Ca^{2+} currents inactivate rapidly and make little contribution to whole-cell current measured at the end of 100–200 ms voltage steps.

Cromakalim-induced current (I_{crom}) was measured as the difference between whole-cell current at the end of 200 ms voltage steps in the absence and presence of cromakalim (100 μ M). The most prominent response to cromakalim, observed in each of more than 90 guinea-pig ventricular myocytes, is a very large outward current at potentials positive to the K^+ reversal potential (Figure 1). In the experiments

described here, outward current measured at +80 mV in the presence of 100 μ M cromakalim (a concentration that induces an effect ~80% of maximal) was 4.4 ± 0.5 nA (range 1.5–12.5 nA, 31 cells) while under control conditions it was 0.65 ± 0.03 nA, in good agreement with previous reports (Escande *et al.*, 1988; Sanguinetti *et al.*, 1988). Mean outward I_{crom} slope conductance (measured between –20 and +20 mV) appeared to increase with $[K^+]_o$ and was: 20.8 ± 2.8 nS for 1 mM K^+ (9 cells), 26.7 ± 6.0 nS for 2.5 mM K^+ (18 cells), 40.0 ± 5.4 nS for 5.4 mM K^+ (14 cells) and 81.9 ± 21.4 nS for 12 mM K^+ (9 cells) as shown in Figures 1 and 2. Only the data from the 12 mM K^+ group were statistically significantly different ($P < 0.01$) from the other groups.

Clear reversal of (inward) I_{crom} was less reliably observed: in 4 of 9 cells in 1 mM K^+ , 9 of 18 cells in 2.5 mM K^+ , 12 of 14 cells in 5.4 mM K^+ , and 7 of 9 cells in 12 mM K^+ . Nevertheless, the reversal potential of I_{crom} in cells in which reversal was observed varied with $[K^+]_o$ concentration strictly as predicted by the Nernst equation: 1 mM $K^+ = -131.5 \pm 1.8$ mV, 2.5 mM $K^+ = -104.6 \pm 6.9$ mV, 5.4 mM $K^+ = -87.2 \pm 1.9$ mV, 12 mM $K^+ = -65.8 \pm 2.2$ mV (Figures 2 and 3a). Inward I_{crom} amplitude was roughly proportional to the $[K^+]_o$ (Figure 2b). Mean inward I_{crom} slope conductance was: 5.3 ± 0.9 nS for 1 mM K^+ , 5.8 ± 0.9 nS for 2.5 mM K^+ , 9.3 ± 1.8 nS for 5.4 mM K^+ and 11.7 ± 4.0 nS for 12 mM K^+ (measured between –150 and –130 mV). In nominally K^+ -free medium (all K^+ -free replaced by Na^+), no inward I_{crom} was measured in any of 5 cells tested while mean outward current slope conductance measured between –20 mV and +20 mV was 25.8 ± 9.2 nS.

Figure 1 Membrane currents recorded from freshly dispersed adult guinea-pig ventricular myocytes under whole-cell clamp. Voltage steps (200 ms) to –200 mV (a1 and a2, only), –150 mV, –100 mV, –50 mV, 0 mV and +50 mV from a holding potential (V_H) of –100 mV were made under control conditions (column 1) and in the presence of cromakalim (100 μ M; column 2) with the external K^+ concentration ($[K^+]_o$) adjusted to 1 mM (a), 2.5 mM (b) or 12 mM (c).

Figure 2 Effects of changes in external K^+ concentration ($[K^+]_o$) on cromakalim-induced current (I_{crom}). (a) Whole-cell current measured at the end of 200 ms voltage steps (in Figure 1) is plotted versus membrane potential under control conditions (open symbols) and in the presence of cromakalim (100 μ M; filled symbols) for four $[K^+]_o$: 1 mM (Δ , $\blacktriangle$), 2.5 mM ($\circ$, $\bullet$), 5.4 mM ($\square$, $\blacksquare$) and 12 mM ($\diamond$, $\blacklozenge$). Data are from the three cells in Figure 1, as well as one not illustrated, in which clear reversal of I_{crom} was evident. (b) I_{crom} , estimated as the difference between whole-cell current measured as in (a) in the absence and presence of cromakalim, is plotted versus membrane potential for the four $[K^+]_o$ (same symbols as in a). Data from 6 to 14 cells.

Figure 3 (a) Plot of the reversal potential of I_{crom} with various external cation concentrations ($[X^+]_o$). The data illustrated are for media containing only K^+ (1, 2.5, 5.4 or 12 mM; ●), only Rb^+ (12 mM, ▲), only Cs^+ (12 mM, ◆), K^+ (2.5 mM) plus Rb^+ (1–30 mM; △) and Rb^+ (12 mM) plus Cs^+ (0.1–30 mM, ◇). Strict adherence to values predicted by the Nernst equation (line) were observed for media containing K^+ and/or Rb^+ . The dotted line adjacent to the 12 Rb^+ /1–30 Cs^+ data illustrates predicted values of I_{crom} reversal potential using the upper limit of $P_{Cs^+}/P_{K^+} = 0.18$ obtained from the reversal of I_{crom} in 12 mM Cs^+ medium. The solid line was drawn using $P_{Cs^+}/P_{K^+} = 0.07$. Data from 4–22 cells. (b) Under the same experimental conditions as in (a), inward I_{crom} amplitude (measured 50 mV negative to E_{rev}) is plotted as a function of $[X^+]_o$. Inward I_{crom} in media containing K^+ and/or Rb^+ showed no sign of saturation over this range of $[X^+]_o$.

Permeabilities of rubidium and caesium

The permeabilities of Rb^+ and Cs^+ relative to K^+ were assessed in terms of their abilities to carry inward cromakalim current and the reversal potential in nominally K^+ -free medium containing either 12 mM Rb^+ or 12 mM Cs^+ .

In medium containing 12 mM Rb^+ prior to application of cromakalim, Na^+ , Ca^{2+} and outward K^+ currents displayed little change in amplitude and voltage-dependence (Figure 4a). I_{K1} was reduced as much as 90% relative to that observed in 12 mM K^+ in a manner consistent with reports of the low Rb^+ permeability of inwardly rectifying K^+ channel of cardiac muscle (Matsuda *et al.*, 1989), skeletal muscle (Standen & Stanfield, 1980) and starfish eggs (Hagiwara & Takahashi, 1974). In the presence of external Rb^+ (12 mM), the average outward I_{crom} amplitude was almost as large as that observed in 12 mM K^+ (Figure 4c and 4d).

However, clear reversal of I_{crom} was observed in all 22 cells recorded in 12 mM Rb^+ and average inward I_{crom} amplitude was virtually identical to that observed in 12 mM K^+ . The average reversal potential (-64.2 ± 1.1 mV) was positive to the average value in 12 mM K^+ by 1.6 mV. Using the relation-

$$\Delta E_{rev} = E_{Rb^+} - E_{K^+} = RT/F \ln (P_{Rb^+}/P_{K^+}) \quad (1)$$

and assuming that intracellular permeant ion concentration is constant and that Rb^+ and K^+ are the only permeant extra-

cellular ions, the permeability of Rb^+ relative to K^+ (P_{Rb^+}/P_{K^+}) is estimated to be 1.06.

In medium containing Cs^+ (12 mM), inward current was reduced to <5% of control at membrane potentials negative to -100 mV (Figure 4b1); Na^+ and Ca^{2+} currents were relatively unaffected. Under these conditions, cromakalim (100 μ M) induced outward current at membrane potentials above -100 mV that was of amplitude comparable to that seen in K^+ -containing or Rb^+ -containing medium at potentials positive to -50 mV (Figure 4). However, in 12 mM Cs^+ medium there was little or no difference in current at membrane potentials below -120 mV in the absence or presence of cromakalim. Clear inward I_{crom} was observed in only 4 of 8 cells. The mean I_{crom} reversal potential in these 4 cells was -110.3 ± 6.6 mV. Using the relationship for Cs^+ permeability analogous to equation (1) an upper limit for the permeability of Cs^+ relative to K^+ (P_{Cs^+}/P_{K^+}) of 0.18 was calculated.

Interactions of Rb^+ and K^+ in the cromakalim-activated channel

Rb^+ (1–30 mM) was added to normal medium containing 2.5 mM K^+ to examine possible interactions between the two permeant cations in the channel activated by cromakalim. Figure 5 illustrates I_{crom} measured under these conditions using the same procedure as in Figures 2b and 4d. Inward I_{crom} amplitude increased with increasing concentrations of Rb^+ (see also Figure 3b), while the slope conductance of outward I_{crom} was unaffected. The I_{crom} reversal potential was shifted to more positive voltages in higher concentrations of Rb^+ in a manner consistent with the relation:

$$E_{rev} = RT/F \ln \{([K^+]_o + P_{Rb^+}/P_{K^+}[Rb^+]_o)/145\}$$

using $P_{Rb^+}/P_{K^+} = 1.06$ obtained above and assuming that the concentration of the intracellular permeant cation, K^+ , remains constant at 145 mM.

Effects of Cs^+ on K^+ and Rb^+ current

The experiments illustrated in Figure 4 show that Cs^+ is a poor charge carrier for I_{crom} . To determine if Cs^+ is able to block the I_{crom} carried by K^+ and Rb^+ , I_{crom} was measured in medium containing 2.5 mM K^+ or 12 mM Rb^+ to which 0.001–30 mM Cs^+ was added. Most of these experiments were carried out with medium containing Rb^+ because these conditions permitted the most reliable measurement of inward I_{crom} .

It was particularly important in these experiments to control for the potent ($IC_{50} = 0.01$ –3 mM), voltage-dependent block of I_{K1} by Cs^+ in order to distinguish it from block of inward I_{crom} . In medium containing 2.5 mM K^+ , our results (not shown) were similar to those of previous reports of block by Cs^+ of I_{K1} in cardiomyocytes (Isenberg, 1976; Argibay *et al.*, 1983; Tourneur *et al.*, 1987; Harvey & Ten Eick, 1989) as well as in other tissues (Hagiwara *et al.*, 1976; Gay & Stanfield, 1977). The concentration of Cs^+ inhibiting I_{K1} by 50% (IC_{50}) was strongly voltage-dependent and varied from 12 μ M at -200 mV to >3 mM at -90 mV with an e -fold change in IC_{50} for a 15 mV change in membrane potential. In medium containing 12 mM Rb^+ , Cs^+ was an effective, but slightly less potent, blocker of I_{K1} (Figure 6). IC_{50} varied from 20 μ M at -200 mV to 8 mM at -90 mV with an e -fold change in IC_{50} for a 27 mV change in membrane potential (Figure 8). The equivalent valence of Cs^+ was estimated using the relation

$$z\delta = RT/F[\ln (IC_{50(E1)}/IC_{50(E2)})/(E1 - E2)]$$

where z is the charge of the blocking cation and δ the fraction of the membrane electrical field that Cs^+ traverses to reach its blocking site. $IC_{50(E1)}$ and $IC_{50(E2)}$ are the half-inhibitory concentrations of the blocking cation at two membrane potentials ($E1$ and $E2$) and R , T and F have their usual thermodynamic meanings. In K^+ -containing medium $z\delta$ was approximately 1.54 while in Rb^+ -containing medium $z\delta$ was 0.82.

Figure 4 Rb⁺ and Cs⁺ as charge carriers of cromakalim-induced inward current (I_{crom}). Whole-cell membrane current was measured as in Figures 1 and 2, but in the presence of 12 mM Rb⁺ (a) or Cs⁺ (b); bathing medium was nominally K⁺-free. Holding potential (V_H) was -100 mV; currents for voltage steps from -150 mV to +50 mV at 50 mV intervals are illustrated. (a1) 12 mM Rb⁺; (a2) cromakalim (100 μ M) in 12 mM Rb⁺; (b1) 12 mM Cs⁺; (b2) cromakalim (100 μ M) in 12 mM Cs⁺. (c) Current-voltage relationship for whole-cell membrane current measured at the end of 200 ms voltage steps in Rb⁺-containing (○, ●) and Cs⁺-containing (Δ, ▲) medium under control conditions (open symbols) and in the presence of cromakalim (100 μ M; filled symbols). Symbols for current in Cs⁺ in the absence and presence of cromakalim overlap at potentials between -200 and -100 mV. (d) Plot of I_{crom} (estimated as in Figure 2) versus membrane potential recorded in 12 mM Rb⁺ (○, 22 cells), 12 mM Cs⁺ (Δ, 8 cells) and 12 mM K⁺ (◇, 9 cells, same data as in Figure 2b).

Cs⁺ also inhibited inward I_{crom} carried by K⁺ and Rb⁺, but had little effect on outward I_{crom} amplitude at membrane potentials more than 50 mV positive to the I_{crom} reversal potential (Figures 6 and 7). In Rb⁺-containing medium with moderate concentrations of Cs⁺ (0.1–1 mM, sufficient to inhibit I_{K1}) inward I_{crom} was unchanged, or even slightly increased. Higher concentrations of Cs⁺ (3–30 mM) strongly inhibited inward I_{crom} . This inhibition was voltage-dependent; the potency of Cs⁺ was greater at more negative membrane potentials such that IC₅₀ varied between 4 mM at -200 mV and 20 mM at -120 mV (Figure 8) and Cs⁺ was entirely ineffective at membrane potentials positive to the reversal poten-

Figure 5 Interactions of K⁺ and Rb⁺ in the cromakalim-activated channel. With the K⁺ concentration ($[K^+]_o$) constant at 2.5 mM, I_{crom} was measured with Rb⁺ concentrations ($[Rb^+]_o$) of 0 mM (●), 1 mM (□), 3 mM (Δ), 10 mM (▽) and 30 mM (◇). Note the positive shift in I_{crom} reversal potential increased inward I_{crom} with increased $[Rb^+]_o$. Data from 4 cells; s.e.mean (not shown) were <20%.

Figure 6 Effects of extracellular Cs⁺ on I_{crom} , I_{K1} and other currents in a ventricular myocyte recorded in 12 mM Rb⁺-containing medium. Voltage (top) and current traces are illustrated for voltage steps from a holding of -100 mV to test potentials of -200 mV and 0 mV under control conditions (a) and in the presence of 100 μ M cromakalim (b). Under control conditions, Cs⁺ (3 mM) blocks I_{K1} activated at -200 mV but has no effect on outward current or inward Na and Ca currents activated at 0 mV. In the presence of cromakalim, inward current at -200 mV is reduced by 3 mM Cs⁺ and blocked by 10 mM Cs⁺. Outward I_{crom} at 0 mV is unaffected by 10 mM Cs⁺.

Figure 7 Effects of extracellular Cs^+ on I_{crom} in 12 mM Rb^+ . (a) As previously, I_{crom} was estimated as the difference between whole-cell membrane current measured in the absence and presence of cromakalim ($100 \mu\text{M}$) in bathing solutions containing 0.01–30 mM CsCl ($[\text{Cs}^+]_o$: 0 mM, ●; 0.1 mM, △; 1 mM, ◇; 10 mM, ▽; 30 mM, ○). Rb^+ (12 mM) was present throughout as the carrier of inward I_{crom} . In (b) I_{crom} (I) measured in the presence of various $[\text{Cs}^+]_o$ is expressed as a percentage of control I_{crom} (I_{max} , $[\text{Cs}^+]_o = 0 \text{ mM}$) and plotted as a function of membrane potential. Same symbols as in (a). (c) I_{crom} data from (a) for different membrane potentials (–200 mV, ●; –170 mV, ▲; –140 mV, ◆; –110 mV, ■) are plotted as a function of $[\text{Cs}^+]_o$. Data from 3–8 cells; s.e. mean of 8–30% are omitted for clarity.

Figure 8 Voltage-dependence of inhibition of I_{K1} and inward I_{crom} by external Cs^+ . The concentration of external Cs^+ that reduced the currents by 50% (IC_{50}) is plotted as a function of membrane potential for I_{K1} recorded in medium containing 2.5 mM K^+ (△) and 12 mM Rb^+ (○) and for I_{crom} recorded in 12 mM Rb^+ medium (●). The lines, through the data were drawn by eye and have slopes of 16, 28 and 44 mV/e-fold change in IC_{50} , respectively.

tial. The equivalent valence $z\delta$ for Cs^+ block of I_{crom} over the voltage range –200 mV to –100 mV was estimated to be 0.46. The reversal potential of I_{crom} in solutions containing 12 mM Rb^+ plus 1–30 mM Cs^+ varied in a manner consistent with the estimate of $P_{\text{Rb}^+}/P_{\text{K}^+}$ (1.06) obtained above and $P_{\text{Cs}^+}/P_{\text{K}^+}$ equal to 0.07 (Figure 3a).

Discussion

This paper describes the ionic selectivity of whole-cell current activated by cromakalim (I_{crom}) in guinea-pig isolated ventricular myocytes. Cromakalim is known to activate ATP-sensitive K^+ [K(ATP)] channels, and correspondingly the sulphonylurea, glibenclamide, inhibits I_{crom} as it does the K(ATP) channel (Schmid-Antomarchi *et al.*, 1987; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988; Sturgess *et al.*, 1988). Our present data support the contention that the main current response to cromakalim is the activation of K(ATP) channels.

In the majority of cardiomyocytes, there appeared to be a decrease in the amplitude of the voltage-dependent Na^+ and Ca^{2+} currents (I_{Na} and I_{Ca}) and (or) a shift of the activation of these currents to more positive potentials. This effect was extremely variable between cells, but was always reversed by glibenclamide (J.C.R. Randle and S.H.R. Olet, unpublished observations and see also Liu *et al.*, 1990). We do not have compelling evidence from our current experiments that this phenomenon is a primary effect of cromakalim on cardiomyocytes as suggested in vascular smooth muscle cells (Okabe *et al.*, 1990). Rather, as described recently by Shuba *et al.* (1990), deviations of true membrane potential from command potential under voltage clamp owing to large membrane currents may be responsible for the changes in I_{Na} and I_{Ca} that we observe.

Cation permeabilities

The reversal potential of I_{crom} varied with $[\text{K}^+]_o$ strictly as predicted by the Nernst equation and no inward I_{crom} was observed when no K^+ was added to the external medium. Thus, under physiological conditions cromakalim activates a pure K^+ conductance in cardiac myocytes, in agreement with the estimates of relative Na^+ and K^+ permeabilities ($P_{\text{Na}^+}/P_{\text{K}^+}$) of the K(ATP) channel in heart (Takei *et al.*, 1985), in skeletal muscle (0.015; Spruce *et al.*, 1987) and in pancreatic β -cells (0.007; Ashcroft *et al.*, 1989). The reversal potential of I_{crom} activated in the presence of 12 mM Rb^+ or combinations of 2.5 mM K^+ plus 1–30 mM Rb^+ varied in a manner suggesting that Rb^+ and K^+ have similar permeabilities ($P_{\text{Rb}^+}/P_{\text{K}^+} = 1.06$). This value is slightly higher than those obtained in single K(ATP) channel recordings in skeletal muscle (0.76; Spruce *et al.*, 1987) and β -cells (0.7; Ashcroft *et al.*, 1989). Similar experiments with Cs^+ -containing media indicate that Cs^+ has a low, but measurable permeability with $P_{\text{Cs}^+}/P_{\text{K}^+} < 0.2$. The estimate of $P_{\text{Cs}^+}/P_{\text{K}^+} = 0.18$ from experiments with 12 mM Cs^+ must be considered an upper limit since this value was derived from the I_{crom} reversal potential in four cells in which clear reversal occurred, while in four other cells no I_{crom} reversal was observed even at –200 mV. Thus, a $P_{\text{Cs}^+}/P_{\text{K}^+} = 0.07$, which adequately describes the data from experiments in which 1–30 mM Cs^+ was added to 12 mM Rb^+ , is probably closer to a 'true' value. The permeability sequence for monovalent alkali metal cations is therefore $\text{K}^+ \approx \text{Rb}^+ \gg \text{Cs}^+ > \text{Na}^+$.

Inward I_{crom}

As illustrated in previous reports (Sanguinetti *et al.*, 1988; Liu *et al.*, 1990), the amplitude of inward I_{crom} for a given $[\text{K}^+]_o$ was extremely variable, to the extent that even in high $[\text{K}^+]_o$ some cells displayed no inward I_{crom} . This phenomenon is not peculiar to the activation of K(ATP) channels by cromakalim. Activation of K(ATP) channels by depletion of intracellular

ATP in cardiac myocytes can induce a decrease (rather than an increase) in total inward current at negative potentials associated with the appearance of a large outward current at positive potentials (Noma & Shibasaki, 1985).

It is unlikely that the inconsistency in inward I_{crom} observed results from a property of the K(ATP) channel. Single channel studies of K(ATP) indicate that rectification of inward current does not occur. Rather, the inconsistency in the measurement of inward I_{crom} (or $I_{\text{K(ATP)}}$) probably results from technical difficulties associated with the very large inwardly rectifying K^+ current, I_{K1} , in ventricular myocytes. I_{K1} amplitude is typically 5 to 10 times that of the mean inward I_{crom} , so small variations in I_{K1} amplitude will interfere significantly with measurement of inward I_{crom} . Second, small series resistance compensation errors may result in significant errors in the measurement of membrane potential over the range where I_{K1} is active. Finally, the large size of these cylindrical (10–20 μm diameter $\times$ 70–200 μm long) cells, their complex intracellular organization and their high specific membrane conductivity over the range of voltages where I_{K1} is active may conspire to make space clamp imperfect at voltages negative to E_{K^+} (cf. Brown *et al.*, 1981; Shuba *et al.*, 1990). The effects of external Rb^+ and Cs^+ on the amplitudes of inward I_{K1} and I_{crom} also support these conclusions (see below). Furthermore, we have observed consistent reversal of I_{crom} in an insulin-secreting cell line, RIN m5F, which has little active current at voltages negative to E_{K^+} and favourable geometry (unpublished observations).

Our experiments suggest that the conductivities of Rb^+ and K^+ in the cromakalim-activated channel [presumably K(ATP)] of cardiomyocytes are similar and that the two ions do not interfere with each other's passage through the channel. Our results are thus in contrast with single channel data for the K(ATP) channel of skeletal muscle (Spruce *et al.*, 1986; 1987) and pancreatic β -cell (Ashcroft *et al.*, 1989). This may reflect differences in the permeability characteristics of K(ATP) channels of various tissues, in addition to their differing sensitivities to cromakalim, sulphonylureas and ATP (for review see Quast & Cook, 1989b).

Caesium block of I_{K1} and I_{crom}

Our observations of the voltage-dependent block of I_{K1} by Cs^+ in the presence of 2.5 mM external K^+ are similar to those of previous authors (Isenberg, 1976; Argibay *et al.*, 1983; Tourneur *et al.*, 1987; Harvey & Ten Eick, 1989). Interestingly, the Cs^+ block of I_{K1} in the presence of 12 mM Rb^+ was less potent and the voltage-dependence was considerably less than that observed in 2.5 mM K^+ (Figure 8). The equivalent valence $z\delta$ of 1.54 in 2.5 mM K^+ medium agrees with estimates in the range 1.3 to 1.6 made by the aforementioned

authors. Since these estimates are greater than unity, it is proposed that the channel is blocked by two or more Cs^+ ions that traverse a significant portion of the membrane electrical field to reach their blocking sites. The lower equivalent valence of Cs^+ for the block of Rb^+ current through the I_{K1} channel (0.82) may result from differences in interactions between the permeant cations, K^+ and Rb^+ , and the blocking cation, Cs^+ , at the three binding sites proposed for this channel (cf.: Hille & Schwarz, 1978). Further examination of this question is beyond the scope of this paper.

Cs^+ (0.1–30 mM) had no effect on outward I_{crom} . Cs^+ blocked inward I_{crom} carried by Rb^+ considerably less potently than it did I_{K1} . We observed IC_{50} values of 4–20 mM for membrane potentials between –200 and –100 mV and an effective valence of 0.55. Notably, external Cs^+ at concentrations as great as 30 mM had no consistent effect on outward I_{crom} . Quayle *et al.* (1988) reported somewhat greater potency and voltage-dependence ($z\delta = 1.22$) for the Cs^+ block of single skeletal muscle K(ATP) channel current. This discrepancy may reflect differences in the ability of Cs^+ to block the passage of Rb^+ and K^+ in the channel in a manner analogous to the I_{K1} channel. Another possible explanation is that the technical difficulties associated with large-amplitude I_{K1} discussed above interfered with the precise measurement of I_{crom} in the absence of Cs^+ . In fact, moderate concentrations (0.1–1 mM) of Cs^+ that blocked I_{K1} often appeared to increase inward I_{crom} . It seems unlikely that this represents a true increase in I_{crom} since Quayle *et al.* (1988) observed no such increase in single K(ATP) channel current at these concentrations of Cs^+ . With I_{K1} blocked in the presence of Cs^+ , the improved measurement of I_{crom} would have resulted in the apparent increase in I_{crom} and decreased the apparent potency of Cs^+ .

Conclusions

Our results confirm that cromakalim activates a pure K^+ conductance in guinea-pig ventricular myocytes, in support of the contention that K(ATP) channel activation is the major mechanism of action of this benzopyran. The high apparent Rb^+ permeability and conductivity argue that Rb^+ is a good substitute for K^+ in ion flux studies. Rb^+ has also proved to be useful in electrophysiological studies where reliable measurements of inward, as well as outward, I_{crom} are required. Extracellular Cs^+ blocks inward I_{crom} weakly in comparison with its block of the inwardly rectifying current, I_{K1} . Outward I_{crom} is insensitive to extracellular Cs^+ .

We would like to thank Dr G.L. Regnier (Chemistry Department, Institut de Recherche Servier) for providing the cromakalim used in this study and Mrs S. Dumoulin for typing the manuscript.

References

- ARGIBAY, J.A., DUTEY, P., ILDEFONSE, M., OJEDA, C., ROUGIER, O. & TOURNEUR, Y. (1983). Block by Cs of K current I_{K1} and of carbachol induced K current I_{Cch} in frog atrium. *Pflügers Arch.*, **397**, 295–299.
- ASHCROFT, F.M., KAKEI, M. & KELLY, R.P. (1989). Rubidium and sodium permeability of the ATP-sensitive K^+ channel in single rat pancreatic β -cells. *J. Physiol.*, **408**, 413–430.
- BROWN, A.M., LEE, K.S. & POWELL, T. (1981). Voltage clamp and internal perfusion of single rat heart muscle cells. *J. Physiol.*, **318**, 455–477.
- CAVERO, I., MONDOT, S. & MESTRE, M. (1989). Vasorelaxant effects of cromakalim in rats are mediated by glibenclamide-sensitive potassium channels. *J. Pharmacol. Exp. Ther.*, **248**, 1261–1268.
- DIFRANCESCO, D., FERRONI, A. & VISENTIN, S. (1984). Barium-induced blockade of the inward rectifier in calf Purkinje fibres. *Pflügers Arch.*, **402**, 446–453.
- DUNNE, M.J., YULE, D.I., GALLACHER, D.V. & PETERSEN, O.H. (1989). Cromakalim (BRL 34915) and diazoxide activate ATP-regulated potassium channels in insulin-secreting cells. *Pflügers Arch.*, **414** (Suppl. 1), S154–S155.
- ELTZE, M. (1989). Competitive antagonism by glibenclamide of cromakalim inhibition of twitch contractions in rabbit vas deferens. *Eur. J. Pharmacol.*, **161**, 103–106.
- ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.
- GAY, L.A. & STANFIELD, P.R. (1977). Cs^+ causes a voltage-dependent block of inward K currents in resting skeletal muscle fibres. *Nature*, **267**, 169–170.
- HAGIWARA, S., MIYAZAKI, S. & ROSENTHAL, N.P. (1976). Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *J. Gen. Physiol.*, **67**, 621–638.
- HAGIWARA, S. & TAKAHASHI, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J. Memb. Biol.*, **18**, 61–80.
- HAMIL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflü-*

- gers *Arch.*, **391**, 85–100.
- HARVEY, R.D. & TEN EICK, R.E. (1989). Voltage-dependent block of cardiac inward-rectifying potassium current by monovalent cations. *J. Gen. Physiol.*, **94**, 349–361.
- HILLE, B. & SCHWARZ, W. (1978). Potassium channels as multi-ion single-file pores. *J. Gen. Physiol.*, **72**, 409–442.
- ISENBERG, G. (1976). Cardiac purkinje fibers: cesium as a tool to block inward rectifying K^+ currents. *Pflügers Arch.*, **365**, 99–106.
- KAKEI, M., NOMA, A. & SHIBASAKI, T. (1985). Properties of adenosine-triphosphate regulated potassium channels in guinea-pig ventricular cells. *J. Physiol.*, **363**, 441–442.
- LEECH, C.A. & STANFIELD, P.R. (1981). Inward rectification in frog skeletal muscle fibres and its dependence on membrane potential and external potassium. *J. Physiol.*, **319**, 295–309.
- LIU, B., McCULLOUGH, J.R. & VASSALE, M. (1990). On the mechanism of increased potassium conductance by the K^+ channel opener BRL 34915 in isolated ventricular myocytes. *Drug Dev. Res.*, **19**, 409–423.
- MATSUDA, H., MATSURA, H. & NOMA, A. (1989). Triple-barrel structure of inwardly rectifying potassium channels revealed by Cs^+ and Rb^+ block in guinea-pig heart cells. *J. Physiol.*, **413**, 139–157.
- MITRA, R. & MORAD, M. (1985). A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am. J. Physiol.*, **249**, H1056–H1060.
- NOMA, A. & SHIBASAKI, T. (1985). Membrane current through adenosine-triphosphate-regulated potassium channels in guinea-pig ventricular cells. *J. Physiol.*, **363**, 463–480.
- OKABE, K., KAJIOKA, S., NAKAO, K., KITAMURA, K., KURIYAMA, H. & WESTON, A.H. (1990). Actions of cromakalim on ionic currents recorded from single smooth muscle cells of the rat portal vein. *J. Pharmacol. Exp. Ther.*, **252**, 832–839.
- QUAST, U. & COOK, N.S. (1989a). In vitro and in vivo comparison of two channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. *J. Pharmacol. Exp. Ther.*, **250**, 261–271.
- QUAST, U. & COOK, N.S. (1989b). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10**, 431–435.
- QUAYLE, J.M., STANDEN, N.B. & STANFIELD, P.R. (1988). The voltage-dependent block of ATP-sensitive potassium channels of frog skeletal muscle by caesium and barium ions. *J. Physiol.*, **405**, 677–697.
- SANGUINETTI, M.C., SCOTT, A.L., ZINGARO, G.J. & SIEGL, P.K.S. (1988). BRL 34915 (cromakalim) activated ATP-sensitive K^+ current in cardiac muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8360–8364.
- SAKMANN, B. & TRUBE, G. (1984). Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart. *J. Physiol.*, **347**, 641–647.
- SCHMID-ANTOMARCHI, H., DEWILLE, J., FOSSET, M. & LAZDUNSKI, M. (1987). The antidiabetic sulfonylurea glibenclamide is a potent blocker of the ATP-modulated K^+ channel in insulin secreting cells. *Biochem. Biophys. Res. Commun.*, **146**, 21–25.
- SHUBA, Y.M., HEBLINGER, B., TRAUTWEIN, W., McDONALD, T.F. & PELZER, D. (1990). A dual-pipette technique that permits rapid internal dialysis and membrane potential measurement in voltage-clamped cardiomyocytes. *Pflügers Arch.*, **415**, 767–773.
- SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.
- SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1986). Rb^+ permeability of ATP-dependent potassium channels of frog skeletal muscle. *J. Physiol.*, **378**, 105P.
- SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1987). Studies of the unitary properties of adenosine-5'-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.*, **382**, 213–236.
- STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.
- STANDEN, N.B. & STANFIELD, P.R. (1980). Rubidium block and rubidium permeability of the inward rectifier of frog skeletal muscle fibres. *J. Physiol.*, **304**, 415–435.
- STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.*, **95**, 83–94.
- TOURNEUR, Y., MITRA, R., MORAD, M. & ROUGIER, O. (1987). Activation properties of the inward-rectifying potassium channel on mammalian heart cells. *J. Memb. Biol.*, **97**, 127–135.
- WINQUIST, R.J., HEANEY, L.A., WALLACE, A.A., BASKIN, E.P., STEIN, R.B., GARCIA, M.L. & KACZOROWSKI, G.J. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **248**, 149–156.

(Received November 13, 1990

Revised March 12, 1991

Accepted March 18, 1991)